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# RepAM of the *Amycolatopsis methanolica* integrative element pMEA300 belongs to a novel class of replication initiator proteins

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Accessory genetic elements, such as plasmids and integrative elements, are widespread amongst actinomycetes, but little is known about their functions and mode of replication. The conjugative element pMEA300 from *Amycolatopsis methanolica* is present mostly in an integrated state at a single specific site in the chromosome, but it can also replicate autonomously. Complete nucleotide sequencing, in combination with deletion studies, has revealed that *orfB* of pMEA300 is essential for autonomous replication in its host. In this study, it was shown that purified OrfB protein binds specifically to the 3' end of its own coding sequence. Within this short sequence, a putative hairpin structure is located, which contains several direct and inverted repeats, and a nucleotide stretch that resembles the nicking site of the pC194 family of rolling circle replicating plasmids. Additional binding studies revealed that OrfB binds to an 8 bp inverted repeat that occurs three times within the hairpin structure. The data presented show that OrfB is the replication initiator (Rep) protein of pMEA300, and is therefore termed RepAM. Surprisingly, RepAM lacks significant sequence similarity with known prokaryotic Rep proteins, but it is highly similar to a number of yet uncharacterized ORFs that are located on integrative and conjugative elements of other actinomycetes. It is concluded that RepAM and its homologues are members of a novel class of Rep proteins.

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## INTRODUCTION

Actinomycetes are Gram-positive mycelium-forming bacteria that play an important role in mineralization processes, and are abundant producers of antibiotics and other secondary metabolites. Accessory genetic elements, including circular and linear plasmids, and integrative and conjugative elements, are widespread in these organisms. Many elements encode a wide variety of known functions, such as antibiotic resistance, or proteins for unique catabolic pathways (Jacoby & Shapiro, 1977). However, to date, only a single phenotype has been found to be associated with some of the actinomycete plasmids and integrative elements: when plasmid-carrying donor cells are grown on plates together with an excess of plasmid-lacking recipient cells, inhibition zones are formed after plasmid transfer, and these are known as pocks (Bibb *et al.*, 1977).

The small circular actinomycete plasmids and integrative elements for which the replication mechanism has been identified have all been found to replicate via the rolling

circle replication (RCR) mechanism (Hagege *et al.*, 1993). RCR plasmids are divided into five families: pT181, pC194, pMV158 and pSN2 (del Solar *et al.*, 1998), and a recently described family of RCR replicons of *Corynebacterium* spp. (Nesvera *et al.*, 1997; Osborn *et al.*, 2000). Actinomycete RCR plasmids and integrative elements belong either to the pC194 plasmid family (Hagege *et al.*, 1993; Muth *et al.*, 1995; Servin-Gonzalez, 1993; Suzuki *et al.*, 1997), which is widely distributed in Gram-positive and Gram-negative bacteria, or to the fifth family (RCR replicons of *Corynebacterium* spp.). RCR is initiated when the replication initiator (Rep) protein binds to DNA cognate sites, and nicks the plus strand of the double-stranded origin (DSO). The Rep protein is covalently attached to the 5' phosphate at the nick site, while leading strand replication is initiated from the 3' OH end. After the leading strand has been fully displaced, the Rep protein cleaves the displaced ssDNA at the regenerated nick site. A series of cleavage/joining reactions generates a double-stranded plasmid and a circular single-stranded plasmid. The latter is converted into dsDNA using the single-stranded origin and the host replication machinery.

The actinomycete *Amycolatopsis methanolica* harbours a 13.3 kb integrative element, pMEA300. The complete

Abbreviations: DSO, double-stranded origin; ICE, integrative and conjugative element; RCR, rolling circle replication.

sequence of pMEA300 has been determined previously (GenBank accession no. L36679), revealing 20 putative ORFs. Construction and characterization of deletion derivatives has allowed the identification of genes required for replication, regulation, integration and conjugation (Vrijbloed *et al.*, 1994, 1995a, b, c). Previous experiments have suggested the presence of a system for high-frequency spontaneous mutagenesis on pMEA300 (Vrijbloed, 1996). Unfortunately, additional experiments have not been able to substantiate this mutator phenotype.

Based on structural and functional similarity, pMEA300 groups into a class that consists of integrative and conjugative elements (ICEs) (Burrus *et al.*, 2002) of several actinomycetes (Raynal *et al.*, 1998): SLP1 from *Streptomyces coelicolor* A3(2) (Bibb *et al.*, 1981), pSAM2 from *Streptomyces ambifaciens* (Pernodet *et al.*, 1984), pIJ110 from *Streptomyces parvulus* (Hopwood *et al.*, 1984), pIJ408 from *Streptomyces glaucescens* (Hopwood *et al.*, 1984; Sosio *et al.*, 1989), pSG1 from *Streptomyces griseus* (Cohen *et al.*, 1985), pSE101 (Brown *et al.*, 1988) and pSE211 (Brown *et al.*, 1990) from *Saccharopolyspora erythraea*, pMEA100 from *Amycolatopsis mediterranei* (Moretti *et al.*, 1985), and probably pMR2, a plasmid from *Micromonospora rosaria* that has recently been sequenced (Hosted Jr *et al.*, 2005).

pMEA300 is present mostly as an integrated form within a chromosomal gene encoding isoleucine tRNA (Vrijbloed *et al.*, 1994), but it can also replicate autonomously. The region required for replication of pMEA300 has been previously minimized to two unlinked DNA fragments encoding OrfA and OrfB, and KorA (Vrijbloed *et al.*, 1995a). In this paper, we show that OrfB is the Rep protein of pMEA300, and that it has unique DNA-binding properties.

## METHODS

**Bacterial strains and culture conditions.** *A. methanolic* wild-type (NCIB 11946; de Boer *et al.*, 1990), and the plasmid-pMEA300-deficient strain WV1 (Vrijbloed *et al.*, 1994, 1995b), were used. The procedures followed for cultivation in batch cultures, harvesting of cells, and growth measurements, have all been described previously (de Boer *et al.*, 1990). *Escherichia coli* strains JM109 and BL21(DE3) were grown in Luria-Bertani (LB) medium at 37 and 30 °C, respectively.

**DNA manipulations.** *A. methanolic* plasmid DNA was isolated using the Qiagen Plasmid Midi kit, with the following modifications. Cells were harvested from 50 ml YEME (Vrijbloed *et al.*, 1995a), and incubated at 37 °C for 30 min in a cell resuspension buffer (P1), which was supplemented with 4 mg lysozyme ml<sup>-1</sup> (Sigma). After addition of lysis buffer (P2), the mixture was incubated for 15 min at room temperature, and then, after mixing with the protein precipitation solution (P3), it was placed on ice for 30 min. Total DNA was isolated with the Wizard Genomic DNA Purification kit (Promega) from 2 ml of overnight YEME cultures.

Plasmid DNA from *E. coli* was isolated using the QIAprep Spin Miniprep kit (Qiagen). All other DNA manipulations were done according to standard protocols (Sambrook *et al.*, 1989).

**Construction of pMEA300 deletion derivatives.** pMEA300 deletion derivatives used in this study were constructed from the *E.*

*coli*-*A. methanolic* shuttle vector pWV136, which is devoid of integrative and conjugational functions (Vrijbloed *et al.*, 1995a). pHK315 was obtained by removing the remaining transfer genes (*Clal*-*ScaI* fragment) from pWV136. pHK313 is a *SacI*-*ScaI* deletion construct of pWV136, containing a disruption of the *orfA* gene that was created by digestion of the *BstEII*(2) site in *orfA*, and a subsequent Klenow DNA polymerase fill-in reaction, resulting in a frameshift starting at amino acid residue 66 (the complete length of OrfA is 170 aa).

**Transformation of *A. methanolic* WV1.** A simplified version of a previously described method (Vrijbloed *et al.*, 1995b) was used to transform *A. methanolic* WV1. This improved method, which omits the soft agar overlay step, is much less laborious, and yields similar transformation frequencies, as reported previously (between  $3 \times 10^4$  and  $5 \times 10^4$  transformants, using saturating concentrations of  $> 1.0 \mu\text{g}$  plasmid DNA; Vrijbloed *et al.*, 1995b). Additionally, the new procedure significantly reduces background growth on agar plates. In order to prepare competent cells, overnight cultures of *A. methanolic* WV1 on 50 ml trypticase soy medium (BBL) were grown to an OD<sub>430</sub> of 5.0. After centrifugation (5 min, 3600 g), the cells were washed in 25 ml T<sub>10</sub>E<sub>1</sub> (10 mM Tris/HCl, pH 7.5; 1 mM EDTA) and resuspended in T<sub>10</sub>E<sub>1</sub> to an OD<sub>430</sub> of 160. To 100  $\mu\text{l}$  of the cell suspension, 10  $\mu\text{l}$  0.2 M MgCl<sub>2</sub>, 60  $\mu\text{l}$  4.17 M CsCl<sub>2</sub>, target DNA, and T<sub>10</sub>E<sub>1</sub> to a total volume of 20  $\mu\text{l}$ , were added, and mixed by pipetting. Subsequently, 200  $\mu\text{l}$  65% (w/v) PEG-1000 (Koch Light) was added, and gently mixed by pipetting. The transformation mixture was incubated for 40 min at 37 °C. Following incubation, 1 ml T27M [3% (w/v) trypticase soy broth and 7.3% (w/v) mannitol] (37 °C) was added, gently mixed, and centrifuged (1 min, 10 000 g). After a second wash step with 1 ml T27M, cells were resuspended in 500  $\mu\text{l}$  T27M, and incubated for 5–7 h at 37 °C in a shaking incubator. Finally, an appropriate amount of cell suspension was transferred to T27M agar plates containing kanamycin (15 or 20  $\mu\text{g ml}^{-1}$ ). Transformants appeared after approximately 3 days of incubation at 37 °C.

**Analysis of autonomous replication.** Autonomous replication of the plasmid deletion derivatives in *A. methanolic* WV1 was checked by Southern hybridization of untreated total DNA and plasmid preparations, and on total DNA and plasmid preparations that had been digested with appropriate restriction enzymes. Southern hybridization was performed with a DIG DNA Labelling and Detection kit (Roche Diagnostics) using the *orfB* (*repAM*) gene of pMEA300 as a probe.

**Construction of RepAM overexpression plasmids.** The *repAM* gene was PCR-amplified from pMEA300 plasmid DNA using the following primers: 5'-GCGCATATGACCGCCAACCCCGGAGC-3' and 5'-CGCGGATCCTCAGGCGTTGTGCCGACGAC-3'. The first primer introduces an *NdeI* restriction site (underlined) at the start codon, and the second primer introduces a *BamHI* site (underlined) at the termination codon. Reactions were performed with Vent DNA polymerase (New England Biolabs). Restriction-digested PCR fragments were cloned into the *NdeI*-*BamHI* sites of pET15B (Novagen), introducing an N-terminal His<sub>6</sub> tag in the protein, yielding the expression plasmid pHisRepAM. Sequencing of the pHisRepAM construct (GATC Biotech) showed that no amplification errors had been introduced.

**Overexpression and purification of RepAM protein.** *A. methanolic* pMEA300 RepAM protein was overexpressed in *E. coli* BL21(DE3)-pHisRepAM. Cells were grown at 30 °C in 50 ml LB medium containing 1 M sorbitol, 2.5 mM betaine, and 50  $\mu\text{g}$  ampicillin ml<sup>-1</sup>. Protein expression was induced by addition of 0.5 mM IPTG at an OD<sub>660</sub> of 0.3, and subsequent incubation at room temperature for 24 h. Cells were harvested by centrifugation, and resuspended in 1 ml 25 mM Tris/HCl, pH 8.5. Cell-free extracts were

obtained by sonication and subsequent centrifugation (1 min, 10 000 g). His-tagged RepAM protein was purified with nickel nitrilotriacetic acid (Ni-NTA) resin (Qiagen), using the protocol of the manufacturer.

**RepAM-binding assays.** Purified His-tagged RepAM protein was mixed with DNA fragments, and incubated for 30 min at 37 °C in 15 µl reaction buffer (10 mM Tris/HCl, pH 8, 5 mM MgCl<sub>2</sub>, 100 mM NaCl, 1 mM 2-mercaptoethanol, 5 mM DTT, and 50 µg BSA ml<sup>-1</sup>). Binding of DNA by RepAM was studied by analysing DNA mobility in 1.2 % (w/v) agarose gels. Following electrophoresis, gels were stained with ethidium bromide and photographed under UV illumination. The oligonucleotide probes used in the assay to determine the binding specificity of RepAM to the conserved 8 bp repeat were as follows: 5'-GGGCTGCTATCCTCTAGCGCGTACC-TATGAACGTGTCCCAGGC-3', and 5'-GGGCTGCTATCCTCTAG-CCTTGAAGTGTATGAACGTGTCCCAGGC-3'. dsDNA probes were obtained by mixing equivalent amounts of the 43-mer oligonucleotides with their complementary strand, heating to 95 °C for 10 min, and then slowly cooling to room temperature.

## RESULTS AND DISCUSSION

### *orfB* encodes the replication initiator protein of pMEA300

In a previous study, we found three ORFs to be involved in replication of pMEA300: *korA*, *orfA* and *orfB* (Vrijbloed *et al.*, 1995a). Deletion of the *korA* gene resulted in loss of replication in *A. methanolica*. The *korA* gene product probably has a regulatory function in replication, as its amino acid sequence shows extensive similarity to proteins of the GntR family of transcriptional repressors (Vrijbloed *et al.*, 1995a). Deletion or disruption of *orfB* completely abolishes replication in *A. methanolica* (Vrijbloed *et al.*, 1995a). Although *orfA* is located in an operon directly upstream of *orfB*, disruption of *orfA* (construct pHK313) did not lead to loss of autonomous replication, since free circular pHK313 DNA could still be isolated from the transformants (this study, data not shown). This leaves *orfB* as the sole candidate to encode the Rep protein. Similar to the position of the gene encoding the Rep protein (*repSA*) on pSAM2 (Hagege *et al.*, 1994), *orfB* is located directly upstream of the genes encoding the excisionase (*xis*), the integrase (*int*), and the chromosome attachment site (*attP*). This conserved arrangement of *rep*, *xis*, *int* and *attP* seems to be a general feature of the actinomycete ICE class. Although no sequence similarity has been found between OrfB and RepSA, the deletion studies and the conserved location substantiate our conclusion that OrfB is the Rep protein of pMEA300. Accordingly, *orfB* and its product are referred to as *repAM* and RepAM, respectively. Interestingly, RepAM of pMEA300 shows no similarity to any known bacterial Rep protein. However, RepAM is highly similar to uncharacterized ORFs of two integrative elements: pSE211 (18.1 kb) of *Sac. erythraea* (Brown *et al.*, 1990), and pMEA100 (23.7 kb) of *A. mediterranei* (Madon *et al.*, 1987; unpublished data), which is currently being sequenced by our group. These two elements also belong to the actinomycete ICE class, and their *repAM* homologues are also located directly upstream of *xis*,

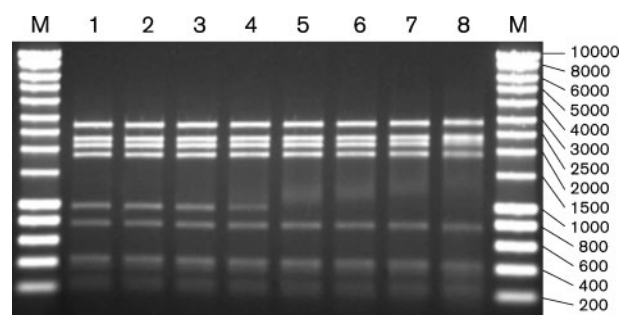
*int* and *attP*. This suggests that these proteins also function as Rep proteins.

### RepAM binds to the 3' end of its own coding sequence

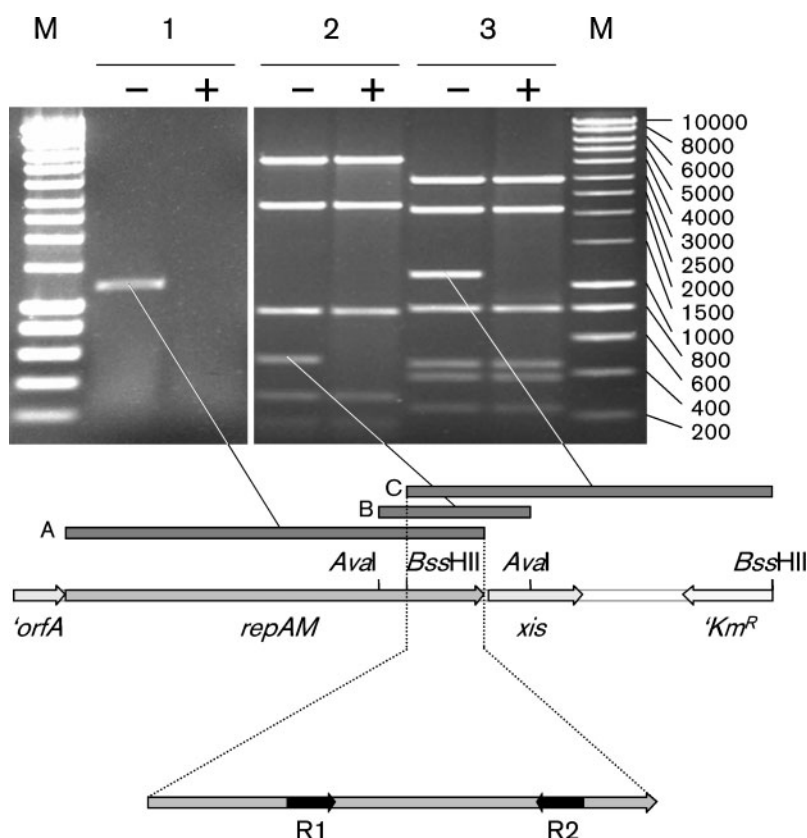
Initiation of plasmid replication often involves binding of a plasmid-encoded replication protein to DNA cognate sites in the origin of replication (*ori*) of the plasmid (Espinosa *et al.*, 2000). To determine whether RepAM is able to bind pMEA300 DNA, binding assays were performed with purified His-tagged RepAM protein, and pMEA300-derived DNA fragments. To obtain RepAM protein, the *repAM* gene was cloned into the expression vector pET15b, and transformed to *E. coli* BL21(DE3). Cell extracts from *E. coli* transformed with pHisRepAM revealed an additional band of approximately 48 kDa on SDS-PAGE gels, which was in agreement with the expected size of RepAM (45.3 kDa). RepAM was purified from the cell extracts using Ni-NTA column chromatography.

*Bss*HII-restricted pMEA300 plasmid DNA was incubated with different concentrations of RepAM protein. Fig. 1 shows that addition of RepAM caused a shift in mobility of the 1007 bp *Bss*HII fragment. This fragment contains the 3' part of *repAM*, the complete *xis* gene, and the first part of the *int* gene. The extent of retardation depended on the concentration of RepAM, since increasing amounts of RepAM gradually decreased the mobility of the bound DNA fragment. This indicates that either the bound DNA fragment contains more than one binding site for RepAM, or RepAM binds to this DNA fragment as a multimer.

To determine the RepAM-binding region more precisely, an *Ava*I/*Bss*HII digest of pHK315 and the *repAM* gene amplified by PCR were used in the gel retardation assay (Fig. 2). Addition of purified RepAM protein prevented migration of the *repAM* PCR fragment, the 454 bp *Ava*I fragment, and the 1096 bp *Bss*HII fragment of pHK315. The RepAM-bound DNA did not migrate through the agarose gel, but



**Fig. 1.** Binding of different concentrations of His-tagged RepAM protein (lanes: 1, 0; 2, 2.3; 3, 4.5; 4, 9.5; 5, 19; 6, 38; 7, 75; and 8, 150 ng) to 0.15 µg *Bss*HII-digested pMEA300 DNA, as shown by agarose gel electrophoresis. M, SmartLadder DNA molecular mass marker (Eurogentec).



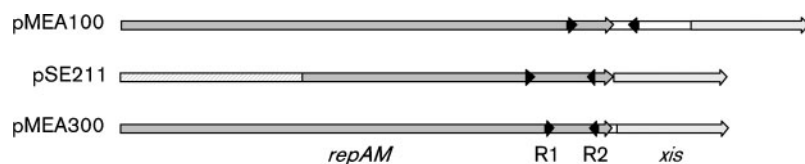
**Fig. 2.** RepAM-binding assays performed with (+) and without (–) 200 ng purified His-tagged RepAM protein, and with 0.02 µg of the PCR product of *repAM* (1), an *Ava*I digest of pHK315 (0.5 µg) (2), and a *Bss*HII digest of pHK315 (0.5 µg) (3). The positions of the bound *repAM* PCR product (A) and restriction fragments (B, C) on pHK315 are indicated. The locations of the 23 bp inverted repeats (R1, R2) of the hairpin structure within the RepAM-binding region are depicted by black arrows.

remained in the slots, which could be observed by ethidium bromide DNA staining, and by Coomassie brilliant blue protein staining (not shown). In this way, we were able to narrow down the RepAM-binding site to a 237 bp DNA fragment; therefore, the putative origin of replication locates within the 3' end of the *repAM* gene. No retardation was observed when eluates obtained from *E. coli* BL21(DE3) containing the empty vector pET15b were used, showing that the observed retardation was RepAM dependent.

### The RepAM-binding region contains several inverted repeats

Interestingly, several direct and inverted repeats were recognized within the 237 bp RepAM binding region. These repeats may form a strong putative hairpin structure of 138 bp, with a  $\Delta G$  of  $-57 \text{ kcal mol}^{-1}$  ( $-238.5 \text{ kJ mol}^{-1}$ ). The hairpin starts with a large 23 bp inverted repeat and includes three identical 8 bp inverted repeats

(GCCGTACC). Similar hairpin structures with multiple identical 8 bp inverted repeats were found at the 3' end of the putative replication initiator genes of pSE211 [174 bp;  $\Delta G$ ,  $-67 \text{ kcal mol}^{-1}$  ( $-280.3 \text{ kJ mol}^{-1}$ )] and pMEA100 [178 bp;  $\Delta G$ ,  $-74 \text{ kcal mol}^{-1}$  ( $-309.6 \text{ kJ mol}^{-1}$ )] (Fig. 3). Moreover, all three hairpins contain a consensus sequence that is similar to the nicking site (5'-CTTGAT-3') of the pC194 RCR family of plasmids (Gruss & Ehrlich, 1989) (Fig. 4). These putative nicking sites are directly flanked by the conserved 8 bp repeats. Secondary DNA structures predicted from the hairpin sequences, using the online secondary structure prediction tool mfold (Zuker, 2003), confirmed that the putative nicking sites are located within unpaired regions of the hairpins (Fig. 5). The *in vivo* conformation of the RepAM-binding region, and the conformation of the DNA in our DNA-binding assays, are unknown. The DNA can have a 'normal' linear dsDNA conformation, or it can fold into two predicted hairpin structures – one on the leading strand, and one on the



**Fig. 3.** Location of the hairpin structures on pMEA300, pSE211 and pMEA100 within the (putative) RepAM-binding regions. The large inverted repeats (R1, R2) of the hairpin structures are depicted by black arrowheads. The part of the putative *rep* gene of pSE211, for which the nucleotide sequence is unknown, is hatched.

pC194	CTTAT <b>CTTG</b> AT
φx174	CCCAAC <b>CTTG</b> AT
pSG5	CGCAG <b>CATG</b> CC
pIJ101	AACAC <b>CTTG</b> GG
pJV1	GACGC <b>CTAG</b> GT
pSN22	CGCGC <b>CTTG</b> GG
pSAM2	CGGGC <b>CTTG</b> AT
pGA1	CGATC <b>CTGG</b> AT
<hr/>	
pMEA300 (s)	<u>GGTACGG<b>CTTG</b></u> <u>AGCCGTACC</u>
pSE211 (s)	<u>GGGACGG<b>CTTG</b></u> <u>TGCCGTCCC</u>
pMEA100 (s)	<u>GTTACCGAT<b>GG</b></u> <u>ATCGGTAAC</u>
<hr/>	
pMEA300 (as)	TACGG <b>CTTG</b> AC
pSE211 (as)	GACGG <b>CTTG</b> AC
pMEA100 (as)	TACCGATCC <b>AT</b>

**Fig. 4.** Alignment of the (putative) nicking sites on plasmids belonging to the pC194 family of RCR plasmids. The conserved 8 bp repeats flanking the putative nicking sites of pMEA300, pSE211 and pMEA100 are underlined; s, sense; as, antisense.

lagging strand – resulting in a cruciform structure. Independent of the conformation, the short 8 bp repeats will be paired with either their inverse complement on the lagging strand or their counterpart within the putative hairpin structure, whereas loops are formed only when in the hairpin conformation. If the secondary structure predictions reflect the *in vivo* conformation upon binding of RepAM, then it would be possible for RepAM to access the nicking site for cleavage; this process is known to occur in plasmids that replicate via the RCR mechanism (Khan, 1997; del Solar *et al.*, 1998).

### RepAM requires at least one of the conserved 8 bp repeat sequences for binding

To determine whether RepAM binds a specific sequence within the 138 bp hairpin structure of pMEA300, binding assays were performed with PCR-amplified DNA fragments comprising various regions of the hairpin (Fig. 6). Our results show that the 8 bp inverted repeats play an essential role in RepAM binding, since gel retardation was observed only when the DNA fragments included at least one of the six conserved repeat sequences.

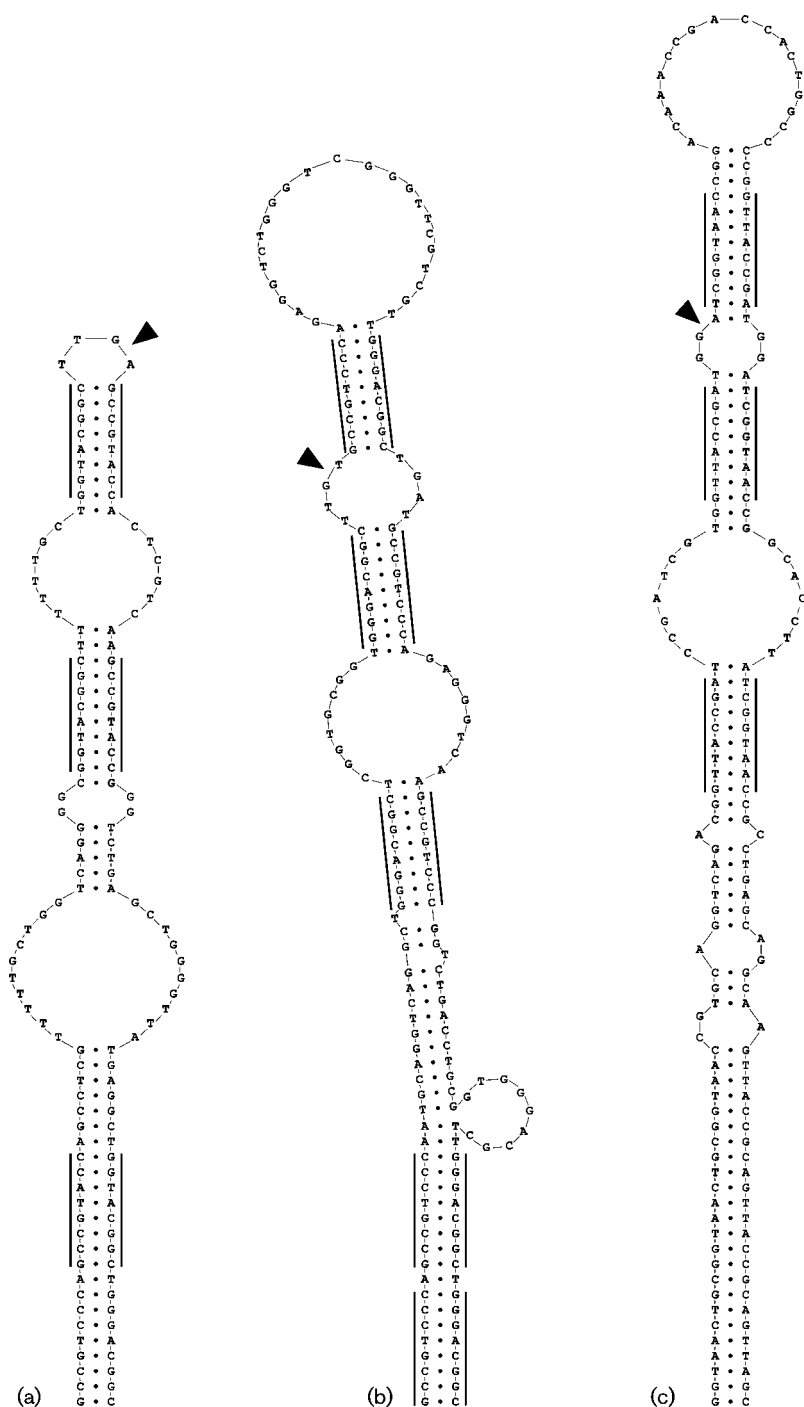
To demonstrate that RepAM indeed binds to these conserved 8 bp repeats, 43-mer double-stranded oligonucleotide probes were designed that had no significant similarity to the 138 bp hairpin sequence, except for the 8 bp repeat sequence, and a derivative was used, in which the eight conserved nucleotides were substituted. In the presence of 500 ng RepAM protein, replacement of the eight conserved nucleotides with other nucleotides caused a significant drop of ~35 % in RepAM-dependent binding. A

residual binding (20 %) of the probe without the conserved 8 bp was most likely to be due to non-specific binding, which is frequently observed for DNA-binding proteins (Lane *et al.*, 1992). These results show that RepAM binds specifically to DNA fragments containing the 8 bp repeat, and thus that the hairpin structure contains multiple RepAM-binding sites.

### RepAM belongs to a novel class of Rep proteins

The DSO region of many RCR plasmids have secondary structures, such as hairpins and cruciforms (Gros *et al.*, 1987; Moscoso *et al.*, 1995; Noirot *et al.*, 1990; Wang *et al.*, 1993). The presence of the hairpin structure at the 3' end of the *repAM* gene, to which the RepAM initiator protein binds, and the presence of a putative nicking site within the hairpin, suggest that pMEA300 also replicates via the rolling circle mechanism. However, the amino acid sequence of RepAM does not share any similarity with known RCR Rep proteins, or with any other Rep protein identified so far. We were also unable to detect any of the consensus sequences characteristic for RCR Rep proteins, such as motifs of the catalytic domain, or those of a putative metal-binding domain (del Solar *et al.*, 1998; Ilyina *et al.*, 1992). Another distinctive feature of pMEA300 is that the putative origin of replication is located within the *repAM* gene itself, at the 3' end. This functional organization differs markedly from other RCR plasmids, in which the DSO is located either upstream of *rep* (pC194, pMV158 and pSN2) or in the proximal part of the *rep* gene, as in the pT181 family (Novick, 1989). A similar atypical distal location of the DSO has been found in the *rep* gene of plasmid pGA1 of the actinomycete *Corynebacterium glutamicum*; this plasmid is a member of the fifth family of RCR plasmids (RCR replicons of *Corynebacterium* spp.) (Abrhamova *et al.*, 2002). However, the Rep proteins of pGA1 and pMEA300 show no significant sequence similarity (<20 %). Furthermore, the sizes of pMEA300 (13.3 kb), pSE211 (18.1 kb) and pMEA100 (23.7 kb) are believed to be too large for a rolling circle mechanism of replication, due to structural instability of large ssDNA intermediates (Helinski *et al.*, 1996). However, ssDNA intermediates can be coated by ssDNA-binding proteins (SSBs), protecting them against nuclease attack and formation of undesired secondary structures (Greipel *et al.*, 1987). Moreover, host-encoded SSBs are known to play a role in replication of plasmids (Helinski *et al.*, 1996; Khan, 1997). Preliminary sequence analysis of pMEA100 has revealed the presence of a unique SSB-encoding gene. Possibly, this additional SSB is required, in addition to chromosomally encoded SSBs, to maintain stability of the 23.7 kb ssDNA intermediate. pMEA300 might be small enough to allow RCR-type ssDNA intermediates to be stabilized by the host SSBs.

In comparison with the Rep proteins of pC194 family of RCR plasmids, RepAM of pMEA300 shares similarity within the putative nicking site only (Fig. 4), and does not contain any of the other conserved motifs found in pC194, or the

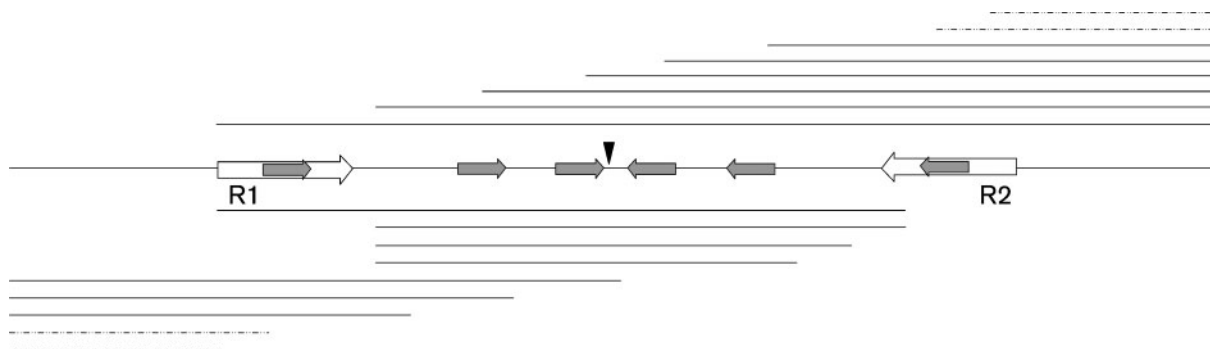


**Fig. 5.** Secondary structures of the hairpins of pMEA300 (a), pSE211 (b) and pMEA100 (c), as predicted by mfold (Zuker, 2003). The conserved 8 bp repeats are outlined by bars. The black arrows indicate the putative nicking sites, which are located in ssDNA regions.

four other families of RCR plasmids (del Solar *et al.*, 1998). Replication proteins and structural features, such as iterons, as found in theta-replicating plasmids (del Solar *et al.*, 1998), are also absent on pMEA300. Therefore, we conclude that RepAM of pMEA300, and its homologues of pSE211 and pMEA100, are members of a novel class of Rep proteins that replicate most probably via an RCR-type replication mechanism. Elucidation of the 3D structure of RepAM could provide important information on the

DNA–protein interactions, and the mechanism of replication, of this novel class of Rep proteins.

In addition to functioning as the origin of replication, the unique location of the RepAM-binding site might have additional effects on the expression regulation of the gene encoding RepAM, or on that of the gene encoding Xis, which is located directly downstream of *repAM*, and is co-transcribed with it.



**Fig. 6.** To determine whether RepAM binds to a specific sequence within the 138 bp hairpin structure of pMEA300, binding assays were performed with PCR-amplified DNA fragments comprising various regions of the hairpin. DNA fragments that were bound by RepAM are represented as continuous lines. The dashed lines represent fragments that were not bound by RepAM. Inverted repeats are shown by arrows (white, 23 bp inverted repeats; grey, 8 bp inverted repeats). The black arrowhead indicates the putative nicking site.

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